Supplemental Material Text S1

Purification of *P. aeruginosa* WaaP

A 996 bp DNA fragment encoding amino acids 1-264 of the *P. aeruginosa* WaaP kinase was amplified from strain PAO1 genomic DNA by PCR using primers waaPEcoRI (5'-GCTCGAATTCTCAGTGATGGTGATGGTGATGACGCTGCTTGCGTTCGTAGAG-3') and waaPNdeI (5'-ATACATATGAGGCTGGTGCTGGAAGAG-3'). These primers were designed to generate a construct with a C-terminal His6 immediately adjacent to amino acid R264. The resulting fragment was digested with *NdeI-EcoRI* and ligated to *NdeI-EcoRI* cut pET21b (Invitrogen). In order to purify the WaaP protein from its native *P. aeruginosa* background and to confirm the purification tag did not render WaaP inactive, an expression system in *P. aeruginosa* was developed as follows. A PCR fragment encompassing the *waaP 1-264 his6* insert from the pET21b construct described above was generated using primers waaPpMMBam (5'-GTTTAAGGATCCGAAGGAGATATACATATGAGG-3') and waaPpMMHind 5'-GCTCAAGCTTTCAGTGATGGTGATGGTGATG-3'). The product was then digested with *Hin*dIII and *Bam*HI and ligated into plasmid pMMB206 (Supplemental Material Table S2) that had been digested with the same enzymes. The resulting construct, pMM-WaaPhis, had WaaP expression under the control of the IPTG inducible *lac* promoter. This construct can replicate in *P. aeruginosa* and was transformed into the *waaP* controlled expression strain CDR0031. Growth of strain CDR0031 harboring pMM-WaaPhis was no longer dependent on induction of the arabinose regulated copy of *waaP* (i.e. the plasmid borne copy complemented the growth defect), demonstrating that the C-terminal His-tagged WaaP was functional (data not shown). For purification, strain CDR0031 (pMMB-waaPhis) was grown in 6 l L-broth supplemented with

chloramphenicol (100 μg/ml), gentamicin (100 μg/ml) and 100 μM IPTG at 37°C overnight with shaking. The cells were collected by centrifugation and frozen at -20°C. 1 g of cell pellet equivalents were typically resuspended in a total volume of 5 ml lysis buffer containing 50 mM Tris pH 8.0, 500 mM NaCl, 50 mM arginine, 50 mM glucose, 20 mM imidazole, 1 mM TCEP, 1 mM PMSF, Complete Protease Inhibitor Cocktail Tablets (1 tablet/50 ml - Roche Biochemicals) and homogenized on ice using a Polytron Mechanical Homogenizer (1 × 30 sec) before being passed through a micro-fluidizer processor (Microfluidics, Newton MA). The lysate was clarified by ultracentrifugation (138,000 × *g*, 60 min, at 4 °C) and passed through a batch/gravity column containing 5 ml volume equivalents of pre-washed IMAC sepharose fast flow resin (GE Healthcare). The resin was washed with 5X column volumes of lysis buffer before bound protein was eluted in a 5X column wash volume containing 50 mM Tris pH 8.0, 500 mM NaCl, 50 mM arginine, 50 mM glutamic acid, 500 mM imidazole, 1 mM TCEP. WaaP-containing fractions were pooled and adjusted to 100 mM NaCl by dilution. At this stage, 1 mM EDTA was added to all subsequent purification steps. WaaP was then further purified from contaminants by ion exchange using Mono-Q and CM-sepharose columns and by size-exclusion chromatography on a Superdex75 column (GE Healthcare). WaaP-containing fraction were pooled and stored at 1.6 mg/ml in 50 mM Tris pH 8.0, 0.5 M NaCl, 50 mM arginine, 50 mM glutamic acid, 1 mM TCEP.

Methods for Lipid A isolation

The pellets from 200- to 1000-ml growths were resuspended up to 20 ml with phosphate-buffered saline (PBS) and transferred to chloroform-safe centrifuge bottles. To the cell suspension, chloroform and methanol were added to form a single-phase Bligh-Dyer solution of chloroform:methanol:aqueous (1:2:0.8, v/v). The solution was stirred for 1 h and then

centrifuged for 20 min at 2,500 × *g* at room temperature. The supernatant containing phospholipids was removed, and the pellet containing LPS was resuspended in 25 ml of 1% acetic acid and transferred to a 50-ml Pyrex glass centrifuge tube. The samples were incubated at 100°C for 1 h with constant stirring to hydrolyze the LPS Kdo glycosidic bonds, thus releasing free lipid A and free core OS. After cooling, the samples were transferred into chloroform-safe bottles containing 25 ml of 0.2 N HCl, 56 ml chloroform, and 56 ml methanol, forming a two-phase Bligh/Dyer mixture (2:2:1.8, v/v). After thorough mixing, the bottles were centrifuged for 20 min at 2,500 × *g* to cleanly separate the phases. The lower (chloroform) phase containing lipid A was removed to a flask and dried in a rotary evaporator. The dried free lipid A was dissolved in 2-4 ml of chloroform:methanol (2:1, v/v) with a bath sonicator and transferred to a screw-cap glass tube with a teflon-lined lid.

Electrospray ionization mass spectrometry (ESI-MS) of lipid A

The ion trap was tuned with the ESI Tuning Mix (Agilent G2431A/G2431-60001) and the quadrupoles tuned with Chemical Standards Kit (ABSCIEX 4406127). To prepare for infusion, lipid A samples were thawed, diluted (generally 10- to 100-fold, as needed) with chloroform:methanol (2:1, v/v) to a volume of 500 µl and piperidine added to a final concentration of 1% (v/v). Each sample was immediately loaded into a Hamilton syringe (#1750) and infused into the source at 10 µl/min with a Pump 11 Plus syringe pump (Harvard Apparatus, Holliston, MA). The parameters were: curtain gas (CUR), 10 psi; ion spray voltage (IS), -4500 V; temperature (TEM), 0; nebulizer gas (GS1), 20 psi; heater gas (GS2), 0; interface heater (ihe), on; declustering potential (DP), -50 to -80 V; entrance potential (EP), -10 V; collision cell exit potential (CXP), -15 V. Nitrogen was the collision gas with the following new parameters:

collision gas (CAD), 5 psi; collision energy (CE), -30 to -55 V; and collision cell exit potential (CXP), -5 V.

High-pressure freeze-substitution TEM

Samples were harvest and fixed as described in Materials and Methods, except that the samples were frozen using a Leica EMPact2 high-pressure freezer (HPF) (Leica, Vienna, Austria). Freeze substitution of the HPF frozen cells was carried out in a Reichert AFS machine (Lecia, Vienne, Austria) in 1% osmium tetroxide plus 0.1% uranyl acetate in acetone at -90˚C for 3 days, then gradually warmed up to 20˚C. Cells were rinsed three times in pure acetone and infiltrated and embedded in TAAB Epon as described for TEM in Materials and Methods.

Supplementary References

1. **Becher A. and H. P. Schweizer**. 2000. Integration-proficient *Pseudomonas aeruginosa* vectors for isolation of single-copy chromosomal *lacZ* and *lux* gene fusions. Biotechniques 29: 948-50, 952.

2. **Dean, C. R., and J. B. Goldberg.** 2002. *Pseudomonas aeruginosa galU* is required for a complete lipopolysaccharide core and repairs a secondary mutation in a PA103 (serogroup O11) *wbpM* mutant. FEMS Microbiol. Lett.**210:**277–283.

3. **Evans, K., Passador, L., Srikumar, R., Tsang, E., Nezezon, J. and K. Poole**. 1998. Influence of the MexAB-OprM multidrug efflux system on quorum sensing in *Pseudomonas aeruginosa*. J. Bacteriol:180:5443-7.

4. **Guzman L. M, Belin D, Carson MJ, and J. Beckwith**. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose pBAD promoter. J Bacteriol.**177**(14):4121-30.

5. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer**. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212**:77-86.

6. **Morales V. M., Backman A., and M. Bagdasarian**. 1991. A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene **97**: 39-47.

7. **Olsthoorn, M. M., J. Haverkamp, and J. E. Thomas-Oates**. 1999. Mass spectrometric analysis of *Klebsiella pneumoniae* ssp. pneumoniae rough strain R20 (O1-: K20-)

lipopolysaccharide preparations: identification of novel core oligosaccharide components and three 3-deoxy-D-manno-oct-2-ulopyranosonic artifacts. J.Mass Spectrom. **34**:622-636.

8. **Schweizer, H. P.** 1993. Small broad-host-range gentamicin resistance gene cassettes for site-specific insertion and deletion mutagenesis. Biotechniques **15**: 831-834.

9. **Simon R., Priefer U., and A. Puhler**. 1983. A broad host range mobilization system for in vivo genetic engineering - transposon mutagenesis in Gram-negative bacteria. Bio-Technology **1**: 784-791.